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Review

Resistance characteristics of influenza to amino-adamantyls

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ABSTRACT

The recent outbreaks of avian flu in Southeast Asia and swine flu in Mexico City painfully exemplify the ability of the influenza virus to rapidly mutate and develop resistance to modern medicines. This review seeks to detail the molecular mechanism by which the influenza virus has obtained resistance to amino-adamantyls, one of only two classes of drugs that combat the flu. Amino-adamantyls target the viral M2 H^+ channel and have become largely ineffective due to mutations in the transmembrane domain of the protein. Herein we describe these resistance rendering mutations and the compounded effects they have upon the protein's function and resulting virus viability.

Contents

1. Influenza

The “Spanish Flu” pandemic of 1918, by some estimates [1], is thought to have killed more individuals than any disease outbreak in recorded human history. Thankfully, recent outbreaks of influenza have not been as deadly, yet it remains a grave health hazard: In the United States, according to the Centers for Disease Control and Prevention, influenza and its complications are currently the leading cause of death due to any infectious disease. Moreover, seasonal epidemics due to antigenic drifts can more than double the mortality rates, while antigenic shifts causing world-wide pandemics may result in far greater casualty levels, as was the case in 1918, 1957 and 1968.

Avian influenza virus has been a cause of much concern [2]. In December of 2003, the largest outbreak of avian influenza due to the deadly H5N1 strain occurred in South East Asia. Although only a limited number of people were infected, it was reminiscent of the Spanish Flu of 1918, in that most deaths occurred in otherwise young, healthy individuals. In this and other avian influenza outbreaks, bird culling was immediately implemented, altogether leading to an estimated slaughter of more than 100,000,000 birds, in an attempt to curb the spread of avian influenza.

More recently, the emergence of a swine flu pandemic from Mexico City has been a cause of much concern [3]. As of April 25th 2010, 477,593 reported cases have been confirmed in 214 countries, resulting in 17,410 deaths. This H1N1 virus was clinically isolated in April of 2009 and was defined as a phase six pandemic alert, the maximum level in the WHO scale. The virus is an apparent reassortment of four endemic strains of influenza: one from humans, one from birds, and two from pigs [4], a fact that further exemplifies influenza's versatility.

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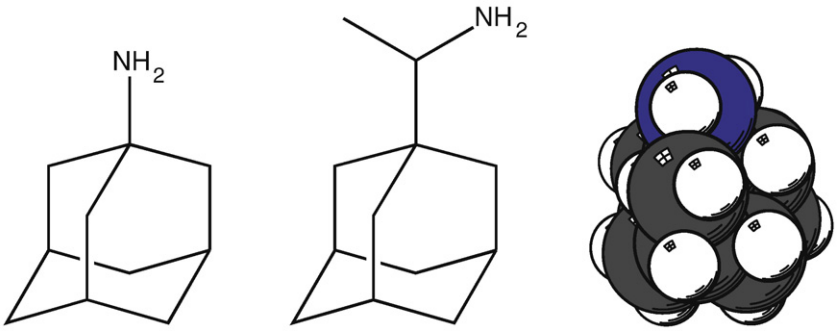

Fig. 1. The structures of the anti-influenza A drugs amantadine (left) and rimantadine (middle). For clarity, only polar hydrogens are depicted. The space-filling representation of amantadine is shown on the right. All structures are shown in their neutral form.

There are three influenza genera in the Orthomyxoviridae family: influenza A, B and C. Type A influenza viruses are further divided into subtypes based on the antigenic relationships in the surface glycoproteins, hæmeagglutinin and neuraminidase. At present, 16 hæmeagglutinin subtypes have been recognized (H1–H16) and nine neuraminidase subtypes (N1–N9). Each virus has one hæmeagglutinin and one neuraminidase antigen, apparently in any combination. All influenza A subtypes in the majority of possible combinations have been isolated from avian species [8]. Still it is important to understand that apart from the antigenic similarity on the surface glycoproteins, the subtypes may contain differences in the rest of the genome. For instance, not all the H1N1 (i.e. that have the same hæmeagglutinin and neuraminidase antigens) have the same characters.

There are two different strategies for combating influenza: vaccination and chemotherapy. Vaccination effectiveness is limited due to the antigenic drifts and shifts that the influenza virus undergoes from year to year. It is for this reason that chemotherapeutic agents are particularly attractive. However, viral resistance to all classes of anti-influenza agents, neuraminidase inhibitors and aminoadamantanes, is found [5]. Moreover, due to reasons that will be elaborated below, aminoadamantanes are effective only against influenza A strains, and are completely ineffective against influenza B strains [6,7].[1]

In this review we will focus on the sequence variations by which influenza develops resistance to amino-adamantyls, shown in Fig. 1. We will begin by describing the major target of amino-adamantyls—the M2 H^+ channel. We will continue by discussing the sequence variations that the M2 protein undertakes and the consequences on its structure and channel activity. We will end by describing some variations in the hæmeagglutinin protein that have been implicated in resistance to amino-adamantyls.

2. The M2 protein

The molecular target of the aminoadamantane drugs, amantadine and rimantadine (the only members of the family approved for prophylactic use) is the influenza A M2 H^+ channel [9]. It is therefore of no surprise, that M2 has been the focus of considerable attention.

The function of M2 was the last step to be elucidated in the life cycle of the influenza virus [10]. Viral attachment and entry into the cell are carried out through the activity of the major viral spike glycoprotein hæmeagglutinin [11]. Membrane fusion and viral genome release occur after hæmeagglutinin undergoes a pH-dependent irreversible conformational change in the acidic endosome. However, it was not clear at first why hæmeagglutinin did not change its conformation in the exocytic pathway where the pH is sufficiently low to cause the conformational change. The answer to this question came upon identifying the pH-dependent H^+ channel activity of M2 [10], which negates the activity of the Golgi H^+ ATPase.

Perhaps more important is M2's role in the virus uncoating process, after viral uptake by endocytosis. The passage of H^+s from the acidic environment of the endosomal lumen into the virus (through M2) weakens the interactions between the matrix protein and the ribonucleoprotein core, enabling the release of the viral genome into the cytoplasm.

3. M2 structure

M2 was shown to be a homo-tetrameric membrane protein [12], stabilized by disulfide bonds [13]. In addition, the transmembrane domain of M2 exhibits ion channel activity and amantadine blockage that is similar to that of the full-length M2 [14]. Taken together, it is clear that tetramerization is initiated by the transmembrane domain and subsequently stabilized by cytoplasmic disulfide bonds.

Several techniques have produced structures for the M2 protein: X-ray crystallography [15], solid-state NMR [16], solution NMR [17] and our 1- and 2D-IR approach [18]. The reader is referred to a recent review on this topic [19]. The X-ray study, in detergent micelles, was of a peptide that encompasses the transmembrane domain of the protein (residues Ser22–Leu46). Furthermore, two structures were obtained: one at pH 7.3 (containing the I33M mutation) and another with amantadine at pH 5.3 (containing the G34A mutation). Both structures were shown to be highly similar to one another. The solid-state NMR experimentally suggested model was also obtained for the same transmembrane peptide, but this time in lipid bilayers [16]. Finally, a solution NMR study of a slightly longer peptide (residues Ser23–Lys60) with rimantadine was reported in detergent micelles, as well [17]. We note that the peptide that was analyzed corresponded to residues Arg18–Lys60, however, the N-terminal five residues were shown to be disordered.

Remarkably, as seen in Fig. 2, there are substantial differences between the various structures: The Cα-RMSD between the X-ray and solid-state NMR structures is 4.01 Å, 4.04 Å between the X-ray and the solution NMR and 4.63 Å between the two NMR structures. The tilt angles of the helices in the solid-state NMR and X-ray structure are similar (30–40°), in contrast to the 23° tilt observed by solution NMR. Furthermore, while the helices in both solution and solid-state NMR structures contact each other throughout, the X-ray structure resembles a conical frustum, with the C-terminal parts splaying apart.

What is most intriguing about the different structures is the location of the drug (see orange molecule in Fig. 2): In the X-ray structure a single amantadine molecule was located in the pore, implying a direct pore blocking model [15]. In contrast, in the solution NMR structure four equivalent allosteric binding sites were found on the exterior of the helical bundle facing the detergent micelle [17]. Moreover, this pocket is located far in the outer region, spanning residues 41–45. The authors postulated that the closed form of the

[1] Aminoadamantanes are ineffective against influenza C strains as well. However, since influenza C virus does not pose a series health threat, all discussion henceforth will be concerned solely with influenza A and B.

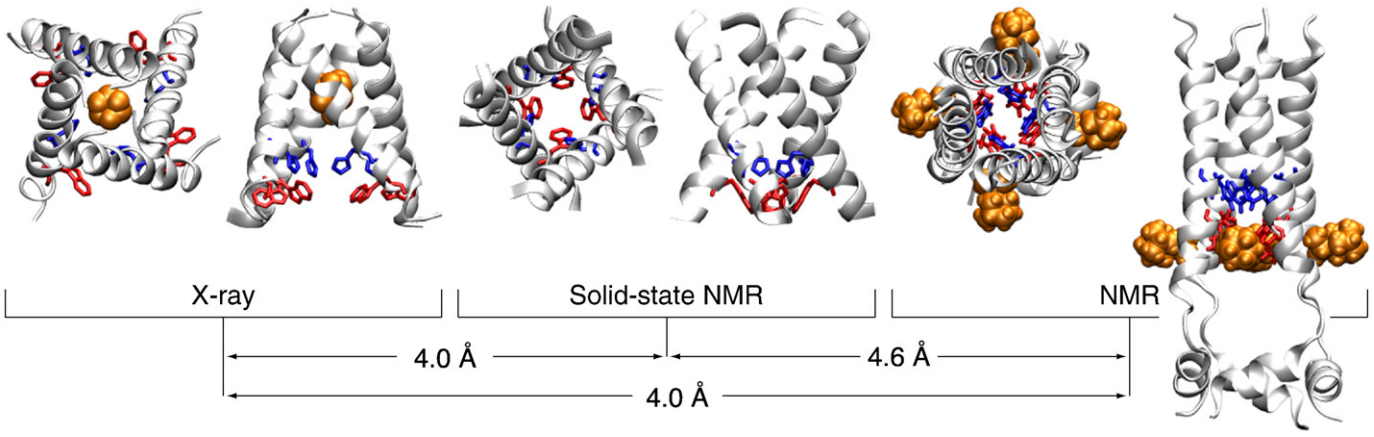

Fig. 2. Structures of the M2 transmembrane domain obtained by three different techniques: X-ray crystallography [15], solid-state NMR [16] and solution NMR [17]. The Cα-RMSDs between the different structures are indicated in the figure. His37 and Trp41 are shown in CPK representation, colored in blue or red, respectively. Amantadine or rimantadine are shown in space-filling representation in gold.

channel is more stable than the open form, whereby drug binding, presumably enforces the energetically favorable closed state. Mutations in the 27–31 spanning region destabilize the complex of the channel and thus shift it to its open state once the pH drops. Finally, the validity of each of these binding sites is a matter of considerable debate [20–22].

4. M2 sequence variations

Whenever influenza viruses are confronted with amino-adamantyls, such as amantadine or rimantadine (Fig. 1), they overcome the resulting M2 channel inhibition by manipulating the sequence of M2 [9]. As seen in Fig. 3 the viral manipulation of the M2 gene is focused in several sites with several variations at each site. A prediction of this particular pattern of resistance mutations is yet to be found.

Since the M2 protein transmembrane segment adopts an α-helical conformation in lipid bilayers [23–25] its residues exhibit a helical periodicity of 3.6 amino acids per turn. This structural arrangement places the amino acids that result in resistance to amino-adamantyls (Fig. 3) in the channel pore and in close proximity to each other. Moreover, this dominant area is suspected as governing either the activity of the channel and/or the binding of amino-adamantyls [15].

When investigating different types of the influenza viruses, one can clearly notice the differences between the avian and human strains of influenza as reported by Hay and colleagues [9]. Hay et al. reported that avian strains such as the A/Chicken/Germany/34 (H7N1 Rostock) and A/Chicken/Germany/27 (H7N7), (Rostock and Weybridge strains, respectively) are inhibited by the application of amantadine prior and post infection. Moreover the mutations that rendered the virus with amantadine resistance at the M2 channel, focused mainly on positions 27, 30 and 34 in the case of the Weybridge strain. In the case of the Rostock strain, the resistance mutations focused on position 27 only. On the other hand, the human strain A/Singapore/1/57 (H2N2) was inhibited when amantadine was implemented prior to infection only. In addition, the resistance acquiring mutations were mainly in positions 30 and 31. Another fact worth mentioning is the variability of mutations per site, which is greater in positions 27, 30 as opposed to site 31 that included one mutation only: S31N [9].

Another report that came more than a decade later [26], showed the same focus on sites 30 and 31, as the exclusive mutations sites causing

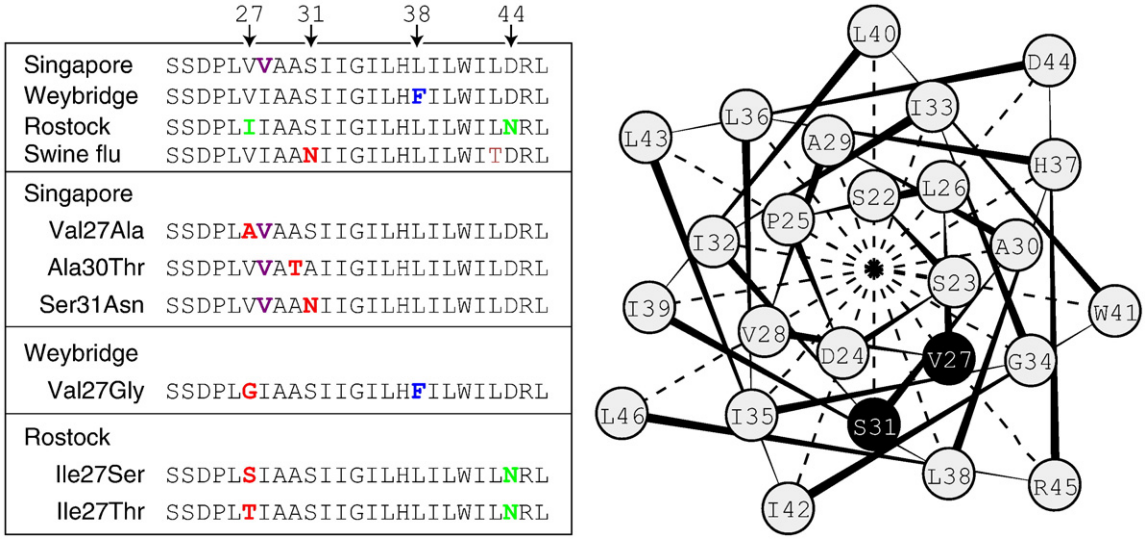

Fig. 3. Sequences of the M2 transmembrane domain. Top panel: differences between the different viral stains. Marked in purple, blue, green and brown are residues unique to Singapore/Udorn, Weybridge, Rostock and swine flu strains, respectively. The red residue in the swine flu (S31N) indicates that it confreres resistance against aminoadamantanes. Note that the transmembrane domain of Udorn strain is identical to that of Singapore. All of the wild-type strains (except the swine flu) are sensitive to amantadine. Bottom panels indicate the different amantadine-resistant mutants and their respective "wild-type" strains. Sites in which differences occur between the mutant, amantadine-resistant strain and its amantadine-sensitive, wild-type parent strain, are marked in red. On the right is a helical wheel diagram (3.6 amino acids per turn) of the Singapore strain indicating the two mutations hotspot positions, 27 and 31.

resistance in the case of H2N2 strain influenza (human influenza strain). The difference in the mutation sites acquiring amino-adamantyl resistance in avian versus human is important, since influenza A strains are mainly transmitted by aquatic birds [27], and the differences may link the possibility of a specific viral genome to cause the next human pandemic.

The mutation site and amino acid substitution may not be random, as shown in a recent study investigating influenza strains that have high potential to cause a pandemic [28]. In these instances the dominant mutation inducing resistance is S31N (e.g.' swine flu as shown in Fig. 3). This study also shows that the mutations occurred mainly after 1983 and were not found prior to that [28,29]. The noted drift toward the S31N mutation once human viruses were sequenced, was also noted at Asian countries around the 2005 avian H5N1 outbreak [30], or even in a more recent study [31]. Thus, an optional scenario is that the S31N mutation, revealed in genomes other than genome B (human origin) is tightly related to strong outbreaks of human influenza, or signals high selective pressure of amino-adamantyls (high usage). Signs of S31N mutations that were linked to major influenza outbreaks were also reported by others, next to the last avian flu outbreak that followed [30,32–34]. Similarly the previous Swine flu attack was followed by the appearance of S31N resistant mutation [35,36].

Another scenario postulates that the appearance of the S31N mutations could all be linked to the emergence of viruses from the same linage, as presented for the A/H3N2 between the years 2004 and 2006 [35], or later in pigs [29]. Interestingly, a recent study from Japan clearly showed that between the years 1999 and 2001, the H3N2 strains gained higher tolerance (compared to H1N1 strains at the same years) against amino-adamantyls [37]. In this instance the resistance was achieved once more by mutating the M2 serine 31 to asparagine (S31N). In contrast, H1N1 assayed viruses that gained lower degree of amino-adamantyl resistance, showed clear preference of the M2 valine 27 to alanine (V27A) mutation. Other reports also marked the difference in the two mutations mentioned above. For instance, in the case of WSN/33 (H1N1), both mutations could bring to amino-adamantyl resistance of the influenza virus, but to a different extent [38]. In this assay, the reported amantadine IC50s of viral plaques infecting MDBK cells, were almost one order of magnitude higher in the case of V27A (WSN/33 (H1N1)) mutated M2 (2012 fold higher than wild-type), versus the S31N mutant (294 fold higher than wild-type). Surprisingly, the weight loss and mortality percentage were slightly higher in the case of the S31N mutant.

5. Mutations in M2 and their effect on activity and stability

If the scenario proposed above is true, then what is the cause for the S31N low IC50 estimate? To answer this question one has to estimate the structural basis of the two mutations. One of the striking differences is of course the size alteration direction of the mutations ("mutation's direction"). In the case of the S31N, the replacement is to a bigger amino acid, and vice versa in the case of the V27A. If both sites are lining the pore or facing inwards, then in the case of mutations following the direction of the S31N mutation, the channel's pore size is most likely reduced. The opposite occurs in the case of V27A, whereby the channel's pore is widened [39]. This observation may be the reason for the changes in the H^+fluxes of the two mentioned mutated M2 ion channels, as viewed in oocytes [10]. Indeed, the S31N mutation applied to the A/Udorn/72- M2 ion channel, showed a decreased current and the V27A showed an increased current of protons through the channel when compared to the wild-type A/Udorn/72- M2 ion channel. A later study calibrated channel activity with protein expression and found that the decrease in conductivity of the S31N mutation was not as high [39]. Finally, this experiment was handled under neutral pH and therefore most of the ion channels is in the closed state, though still leaky and responding to the addition of amantadine [41].

Another noted observation regarding the differences between the two directions of the mutations, is the activity of the mutations. In neutral pH, though the V27A mutation in the A/Udorn/72 M2 ion channel renders the virus amantadine resistant, the channel is still affected by the drug [10]. As a consequence, the addition of 100 μM amantadine causes a decrease of approximately 33% in the channel's activity. That is not the case of the S31N mutated M2 ion channel. Specifically, this particular mutation renders the virus amantadine resistance as well, but under neutral pH it is hardly affected by the drug [10,39].

Later work, explored the influence of amantadine on the very same ion channels, in lower pH conditions [40]. This experiment under conditions in which the channel is mostly in the open state [10,41], showed one order of magnitude higher influx of protons through both mutated channels. Upon the addition of amantadine, the S31N mutated M2 ion channel, showed a decrease in activity of approximately 30%, as opposed to lack of inhibition in the neutral pH. The V27A mutated M2 ion channel showed only 20% decrease of activity as opposed to 33% in neutral pH, as mentioned above [40]. When focusing on the activity levels of the two mutated channels (S31N and V27A), even after taking into account the M2 ion channel's different expression levels, the V27A, mutated M2 ion channel has greater activity in neutral and acidic pH conditions [10,40].

A possible explanation for these observations could be the differences in the binding affinities or the impact of the amino-adamantyls on the different ion channels, when a different pH range is used. When the pH is neutral (7.5), most of the ion channels are either closed or allow only a very low conduction of protons. Since the influx of protons through the S31N ion channel is lower than the V27A mutant [10,40], due to possible narrowing of the pore [39], the addition of amantadine could have little or no influence on the channel at all. When the pH is lowered, the proton flux is increased or the channel's pore is wider and the influence of the amantadine block is much more pronounced. This phenomenon of pH-dependent blocking by amantadine (on the S31N mutated M2 channel) was observed in a later report as well [42]. In the case of the V27A, the proton flux is greater (compared to the S31N case) in any pH [10,40]. Thus, if the addition of amantadine in neutral pH results in a notable (though not full) blocking effect on the channel, when the pH is lowered, the flux is higher and the channel's pore is optionally wider than the optimum for an effective amantadine block.

Another option could be the differences between the binding affinities of amino-adamantyls to the M2 channels in the different pH values. It is also possible that the binding of drug to the S31N mutated ion channel is low in high pH conditions, and is increased in low pH. Accordingly, the V27A mutant could show the direct opposite direction of binding affinity as the pH drops. Nevertheless, it is important to remember that different ion channels are affected differently by the same mutation. A simple comparison of the G34E, A30P and S31N M2 mutations in the different influenza strains Rostock, Weybridge and Udorn [40] or the L38F mutant in the Rostock and Udorn strains [22], clarifies this point.

Taken together it is possible that resistance to amino-adamantyls has an effect on the protein's function and structure. This finding is similar to the effect of mutations on hæmeagglutinin, discussed below in Section 6. Further examples of the resistance mutations are correlated with a decrease in channel's activity, as shown for the A/Udorn/72 (H3N2) M2 mutants: S31N [10], A30P and A30T [40]. It is possible that these observations are responsible for the retarded growth of some resistant viruses. Still as mentioned above, the same mutation can have different impact on different M2 types.

Grambas et al. showed when investigating the A/chicken/Germany/34 (H7N1 Rostock) M2 mutants: A30T, A30E, L26H and S31N, that the growth of these mutated viruses is retarded compared to Rostock wild-type strain [43]. Other mutants such as I27T showed elevated activity compared to the Rostock wild-type channel [43]. Moreover, the authors stated that the impaired growth capacity of certain mutants in chick

embryo fibroblasts was confirmed by their instability to passage, both in the presence and in the absence of amantadine. During passage in the absence of the drug, mutants A30T and G34E readily reverted to the wild-type, i.e. amantadine-sensitive phenotype. The S31N mutant acquired a compensating mutation, I27T which, while retaining drug resistance, conferred improved growth characteristics similar to those of the single mutant I27T. Similarly, passage of A30T and S31N mutants in the presence of amantadine selected variants with the same additional alteration, I27T, and phenotypic characteristics.

The observation of low stability due to impaired growth while acquiring resistance, could explain the presence of sensitive viruses remains, in patients that were treated with amantadine [44]. This report showed that as long as amantadine treatment continued, mutated resistant viruses were formed, yet the wild-type sensitive form of virus was always present, presumably in small titers. Once the amantadine treatment was stopped, the sensitive viruses over-powered the mutated form in those patients.

One intriguing question that was further investigated was the link between the inner radius of the pore and the proton conductance ability of the M2 channel. We speculated once before [39] that the mutation direction (from small residue to large one and vice versa) could influence the channels inner pore radius. In order to link the channel's diameter to its activity, we expressed the three different M2 ion channels in *Escherichia coli* using the pMal-p2x expression system from New England Biolabs (Ipswich, MA) [45]. The expression level that was controlled by the addition of Isopropyl-β-D-thio-galactoside (IPTG), brought to a pronounced decrease in bacterial growth (as observed by OD600).

As presented in Fig. 4, the inhibition was lower in the A/chicken/Germany/34 (H7N1 Rostock) strain compared to the A/Singapore/1/57 (H2N2) M2 ion channel, and was lowest in the S31N mutated A/Singapore/1/57 (H2N2). Since the ion conductance is proportional to the inhibition of growth (higher influx of protons = stronger inhibition), we can learn that the ion conductance is the highest in the wild-type Singapore strain, lower in the Rostock strain and lowest in the Singapore-S31N mutant. The effect of mutation in the Singapore S31N mutant versus the Singapore wild-type is most likely narrowing of the pore [39]. It is also plain to see that the Rostock strain has the same size direction as the Singapore S31N, when compared to the Singapore wild-type. A comparison of amino acids 22–46 that were shown to encompass the M2 transmembrane region [46] between the two strains, reveals three amino acids that differ in the M2 transmembrane region (see Fig. 3). The difference of asparagine 44 in Rostock versus aspartate in Singapore has optionally a little size impact. In contrast,

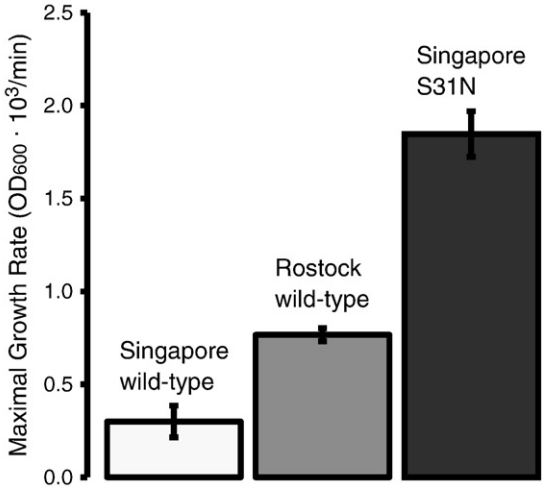

Fig. 4. Activities of different M2 channels, as measured by their effect upon the growth retardation of the host bacteria. Specifically, higher growth rates of the host bacteria indicate weaker channel since it does not have a deleterious effect [45].

sites 27 and 28 contains isoleucines 27 and 28 in the Rostock strain versus valines 27 and 28 in the Singapore strain [9], that has optionally greater size difference, leading to a wider Singapore pore.

It is important to mention the differences between the different ion channels tested by Grambas et al. [43]. The Rostock and Weybridge M2 channels differ not only in their structure and sensitivity to point mutations such as the G34E [43], but also in their activity and ion flux as well. Chizhmakov et al., found that the Rostock M2 ion channel has 5–8 times higher influx of protons than the Weybridge strain [47]. The authors also found that the activity mode of amantadine is slower than predicted by a diffusion limited ligand binding model as did Wang et al. [41]. These authors explained this observation by the possibility of an equilibrium state between the opened and closed channels. Finally the authors state that three mutations are necessary in order to convert the Weybridge M2 to the Rostock strain activity level, while only one is sufficient to do the opposite.

6. Hæmeagglutinin sequence variations

In addition to the influence of amino-adamantyls on the M2 ion channel's sequence, the presence of the drugs has an impact on the hæmeagglutinin protein. As in the case of the amantadine resistance in the M2 protein, the hæmeagglutinin protein was found to contain various mutations in the amantadine resistant viruses [48]. In some cases the mutated hæmeagglutinin that arose by the growth in the presence of amantadine, is sufficient to provide amantadine tolerance [43,49,50]. The amantadine stress has two different levels of action, in low concentrations (0.1–5 μM) and in high concentrations (>100 μM) [9]. Low concentration of amantadine imposes a blockage of the M2 channel in the Trans Golgi Network (TGN). This action results in the decrease of the pH in the TGN that cannot be elevated by the M2 ion channels (since they are blocked). When the pH decreases below a critical level, the hæmeagglutinin proteins are shifted to the low pH form that is responsible for the fusion of membranes [51]. This conformation change is irreversible, and as a consequence high levels of low pH form hæmeagglutinin reach the plasma membrane (as in the case of the Rostock strain) with concomitant impaired release of virions [9,52].

The hæmeagglutinin transformation to its low pH form can take place only after its subunit cleavage [53]. In regards to this fact, it is important to mention that most influenza A hæmeagglutinins are not cleaved intracellularly (as in the case of the Singapore and many other strains) and thus cannot shift to the low pH form in the Golgi [54]. The second route leading to the same result is by the application of amantadine at higher concentrations as mentioned above. This route takes a non specific manner of inhibition [9,48]. The amantadine drug acts as an amino weak base, in high concentrations (>0.1 mM). The molecule has the ability of elevating the lysosomal pH by almost 2 pH units. Amantadine is not the only amine capable of elevating the pH, other amines can impact pH as well [55,56]. Taken together, the direct impact of amantadine treatment is the elevation of resistant viruses with point mutations at the hæmeagglutinin without additional mutations in the M2 ion channel.

As mentioned above, the S31N mutation in various influenza M2 types causes impaired growth [43]. In viral infections such as the Rostock strain, the S31N mutation can also bring about the presence of high levels of low pH form of hæmeagglutinin in the cell plasma membrane [43]. If the virus' inoculum is passed again in the absence of the drug, a compensating mutation such as the I27T reduced the low pH form hæmeagglutinin level to the wild-type level [43]. Interestingly, the authors described the reduced activity of Rostock M2 mutations L26H, A30T, S31N and G34E (A/chicken/Germany/34 (Rostock)). These mutations caused a substantial increase in the expression of the low-pH form of hæmeagglutinin. In addition, sequence changes were observed during subsequent passage of these mutant viruses in the presence or absence of amantadine: reversion to the wild-type form, acquisition of a second suppressor mutation in M2, or the appearance of

a complementary mutation in hæmeagglutinin which increased its pH stability [43]. In contrast, l27T and l27S mutations caused an increase in M2 activity. Furthermore, in double mutants the l27T mutation suppressed the attenuating effects of the A30T and S31N mutations on M2 activity. Finally, in this study, the Rostock hæmeagglutinin mutation hæmeagglutinin2-K58I (a mutation found to decrease viral growth [49]) yielded a decreased pH in which high to low form pH transformation occurred in the hæmeagglutinin. This drop in pH regulates the stability of the hæmeagglutinin that was required by the S31N-K58I double mutant (that has an inhibited M2 flux). Other reports of mutations in the hæmeagglutinin that reduces the high to low pH transformations of the protein are consistent with this study [48], such as the Hemeagglutinin2-G23C mutation [50].

Most influenza viruses, Rostock strain being one exception, are not as sensitive to the Golgi inner pH. When comparing the hæmeagglutinin resistant mutations in other strains (such as the Weybridge influenza strain) to the Rostock strain, the transformation to the low pH form occurs in higher pH values (the opposite) [48,49]. It is possible to rationalize these observations, in that the strains such as Rostock are exposed to the acidification of the Golgi with no relief by the M2 ion channel in the presence of amantadine. Thus the hæmeagglutinin has to be maintained in its high pH form in lower pH conditions, and yet retain fusion capabilities in the late endosome stage. The opposite phenomenon may occur in strains that are not as sensitive to the Golgi pH, since in this case the problem is fusion in the late endosome stage with low acidification condition in the presence of amino-adamantyls. Thus their hæmeagglutinin shifts to its low pH form in higher pH values.

As presented by Daniels et al. the hæmeagglutinin mutations in different influenza strains (X-31 and Weybridge) are focused in different parts of the protein (hæmeagglutinin1 or hæmeagglutinin2) [47]. In this regard, different types of amino acid mutations are taking place in each part: hæmeagglutinin1 mutations are directed towards charged amino acids and hæmeagglutinin2 mutations are from acidic amino acids to neutral ones. Also the amino terminal of the hæmeagglutinin is the area which response to the pH alteration [48].

Finally, the role of hæmeagglutinin in the resistance against amino-adamantyls seems to vary between the different strains. In few cases such as the 2009 reported H3N2 influenza infected patients, the viruses isolated from the patients included mainly M2 mutations. Hæmeagglutinin mutations were hardly present and were only forced out by *in vitro* passages [57]. In other types (H7N7) they are the only cause of resistance [50]. The fusion pH also varies between the different wild-type strains [43,48,49].

7. Mutation rate

It is reasonable to assume that there are different constrains (such as crucial site's structure/function) dominating the mutation rates between different proteins. Thus it is likely that some constrains in addition to the above mentioned, rule the spread of a specific influenza type strain or mutant, over mutations that take place in other viral proteins. This notion can explain the results by Nobusawa and Sato [58]. This assay showed a higher mutation rate in the A types of influenza as opposed to the B types. The authors also presented variations in the mutation rate between the types A (H3N2 versus H1N1) and in the B types as well [58]. Thus it is likely that some mutations are more wide spread in a specific type of influenza, not due to evolutionary stress selection from random occurring mutations, but rather from an indirect reason.

8. Concluding remarks

We have tried to discuss the various mutations that the influenza virus undergoes during its evolution of resistance to amino-adamantyls. The majority of mutations are in the M2 protein which is the prime target of the drug, while some mutations are in the hæmeagglutinin protein mostly due to the impact of amino-adamantyls on the pH. The impact of the resistance mutations on virus viability have been discussed as well, since this has direct ramifications when designing new channel blockers against M2.

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